



Early Journal Content on JSTOR, Free to Anyone in the World

This article is one of nearly 500,000 scholarly works digitized and made freely available to everyone in the world by JSTOR.

Known as the Early Journal Content, this set of works include research articles, news, letters, and other writings published in more than 200 of the oldest leading academic journals. The works date from the mid-seventeenth to the early twentieth centuries.

We encourage people to read and share the Early Journal Content openly and to tell others that this resource exists. People may post this content online or redistribute in any way for non-commercial purposes.

Read more about Early Journal Content at <http://about.jstor.org/participate-jstor/individuals/early-journal-content>.

JSTOR is a digital library of academic journals, books, and primary source objects. JSTOR helps people discover, use, and build upon a wide range of content through a powerful research and teaching platform, and preserves this content for future generations. JSTOR is part of ITHAKA, a not-for-profit organization that also includes Ithaka S+R and Portico. For more information about JSTOR, please contact support@jstor.org.

SOME IMMUNITY REACTIONS OF EDESTIN.*†
THE BIOLOGICAL REACTIONS OF THE VEGETABLE
PROTEINS. III.¹

BENJAMIN WHITE AND OSWALD T. AVERY.

(From the Department of Bacteriology, Hoagland Laboratory, Brooklyn, New York.)

INTRODUCTION.

The study of the immune bodies produced in response to the parenteral introduction of pure proteins has a twofold application. First, by using a pure protein, all the usual accompanying substances present in serum, bacterial masses, etc., such as other proteins, less complex nitrogenous bodies, lipoids, fats and carbohydrates, are eliminated, and the antibodies elaborated may be considered solely as the result of a specific reaction against the protein injected. Therefore these reactions, when analyzed, afford a definite criterion for judging the theories of immunity. Second, the immunity reactions of the pure proteins may serve to establish the biological relationships of various proteins, and possibly to yield information concerning their more intimate chemical constitution and chemical relationships.

The study of proteins of animal origin offers a somewhat limited field, since of all these proteins egg albumin alone can be isolated in a state of purity sufficient for these delicate biochemical tests. The immunity reactions of this substance have been extensively investigated, but the application of the data obtained is necessarily more or less restricted, since it is impossible to prepare comparative proteins of animal origin in as pure a state.

The vegetable proteins, on the other hand, seem to present more advantageous material. Their sources are many, their kinds varied, several of them can be crystallized, and their chemical identities established by the methods developed by Osborne² and

* Received for publication May 17, 1913.

† The expenses of this study were defrayed by a grant from the Committee on Scientific Research of the American Medical Association.

¹ Article I of this series, Wells and Osborne, *Jour. Infect. Dis.*, 1911, 8, p. 66; Article II, Wells and Osborne, *ibid.*, 1913, 12, p. 341.

² *The Vegetable Proteins*, Longmans, Green & Co., London, 1909.

others. Some of them, however, particularly the globulins, introduce discouraging complications, which make the prosecution of the various reactions difficult. The insolubility of the globulins in isotonic salt solution, their strong tendency to precipitate in the presence of serum, and their agglutinating action on the red blood corpuscles are some of the obstacles which are responsible for many experimental failures and for the comparatively scant attention which has been accorded them in immunity studies.

The present study began in an endeavor to obtain controlling evidence for certain other experiments in anaphylaxis which have been carried on in this laboratory for the past two years, hence no attempt has been made to extend the study to any proteins other than edestin. Gliadin has been used merely to test the specificity of the edestin reactions. Naturally, it is highly desirable to institute further controls with other similar proteins and to investigate the immunity reactions of these proteins as well. It is hoped to continue this work in the future.

The preparations used in immunological work obviously must be of the highest possible integrity. Those used in the present study consisted of one of edestin, the globulin from hemp seed (*Cannabis sativa*); and one of gliadin, the prolamins from wheat (*Triticum vulgare*), and were specially prepared by Dr. Thomas B. Osborne for the anaphylactic experiments which have been reported by Dr. H. Gideon Wells and himself. No utensils used in making these particular lots of edestin and gliadin had ever before been employed in preparing proteins, and the most scrupulous care was exercised to meet every possible objection to their mode of preparation.¹

It is unnecessary to review here the literature on the biological and immunological reactions of the vegetable proteins, inasmuch as it has been so thoroughly done by Wells and Osborne.² Further, a detailed description of the antigenic properties of these proteins has recently appeared.³ Various investigators have reported observations on the hemagglutinating properties of some of these sub-

¹ The authors desire to express here their sincere appreciation of Dr. Osborne's many kindnesses and his generosity in supplying them with these two preparations.

² *Jour. Infect. Dis.*, 1911, 8, p. 66.

³ Pick, *Handbuch der pathogenen Mikroorganismen*, Kolle und v. Wassermann, 1912, 1, p. 685.

stances, on the precipitins produced, and on their hyper-sensitive or anaphylactic reactions.

HEMAGGLUTININS.

The agglutinating action of vegetable proteins upon red blood corpuscles has been frequently observed. The crude nature of the extracts employed and the variety of methods used by the various authors make quantitative comparisons difficult. Among the few investigators who employed pure proteins are Osborne, Mendel and Harris,¹ who studied ricin (*Ricinus zanzibarensis*), and Schneider,² working with proteins of the bean (*Phaseolus*). Ricin was found to agglutinate erythrocytes even when added in small amounts and this action is particularly associated with the coagulable albumin of the castor bean. Schneider concluded that the reserve protein of the bean is probably responsible for the agglutinating action, since the action decreases as the seedling develops. Further, the hemagglutinin does not occur in extracts of the root, stems or leaves of the bean plant. These observations are confirmed by those of von Eisler and von Portheim.³ Mendel⁴ has shown that a large number of leguminous seeds possess hemagglutinating properties and that this hemagglutination and toxic action are independent.

It is apparent that various proteins show widely varying degrees of agglutinating action and it has also been found that corpuscles from different animal species exhibit different degrees of sensitivity to this action.

In previous investigations it has been customary to use the proteins dissolved in hypertonic salt solutions and to employ blood-corpuscles in the form of defibrinated blood. In order to exclude any action which might be associated with serum components, the corpuscles in the present experiments were previously washed free from serum. The technic was as follows:

Experiment 82.—The edestin and gliadin solutions were made by rubbing up the dry powdered preparations in a small amount of water, then diluting and adding just enough N/10 sodium hydrate solution to produce solution and finally adding sufficient

¹ *Am. Jour. Physiol.*, 1905, 14, p. 259.

⁴ *Arch. Fisiol.*, 1909, 7, p. 168.

² *Jour. Biol. Chem.*, 1912, 11, p. 47.

³ *Ber. d. deutsch. Botan. Ges.*, 1911, 29, p. 419; *Zentralbl. f. Biochem. u. Biophysik.*, 1911, 12, p. 391.

N/1000 sodium hydrate to give the required concentration of edestin of 1:100. Solutions of 1:1000 and 1:10,000 were made by adding requisite amounts of N/1000 sodium hydrate. The erythrocytes used were sheep's corpuscles washed free from serum and made up in a 1 per cent suspension in isotonic sodium chlorid solution. Sufficient salt solution (1.35 per cent) and water were first put in the tubes to render the final volume of 4 c.c. isotonic, then 1 c.c. of the corpuscle suspension was added to each tube and varying amounts of edestin and gliadin solutions were immediately added slowly, with shaking, in order to prevent the laking action of these hypotonic solutions. Macroscopic and microscopic observations after 15 min. and 7 hrs.

TABLE 1.
HEMAGGLUTINATING ACTION OF EDESTIN AND GLIADIN.

TUBE	EDESTIN			RED CELLS 1% (c.c.)	SODIUM CHLORID SOLUTION 1.35 PER CENT (c.c.)	WATER (c.c.)	AGGLUTINATION	
	Dilution	Quantity (c.c.)	Milligrams				15 min. 37.5°	7 hrs. 2 hrs. 37.5° 5 hrs. 16.0°
1.....	1:100	1.0	10	1.0	2.0		++++	+++++
2.....	"	0.5	5	"	"	0.5	+++	+++++
3.....	"	0.25	25	"	"	0.75	++	++++
4.....	"	0.1	1	"	"	0.9	+	+++
5.....	1:1000	1.0	1	"	"		+	+++
6.....	"	0.5	5	"	"	0.5	=	++
7.....	"	0.25	0.25	"	"	0.75	o	++
8.....	"	0.1	0.1	"	"	0.9	o	+
9.....	1:10000	1.0	0.1	"	"		o	+
10.....	"	0.5	0.05	"	"	0.5	o	o
11.....	"	0.25	0.025	"	"	0.75	o	o
		<i>Gliadin</i>						
12.....	1:100	1.0	10	"	"		o	o
13.....	"	0.5	5	"	"	0.5	o	o
14.....	"	0.25	2.5	"	"	0.75	o	o
15.....				"	"	1.0	o	o

The experiment shows that edestin possesses hemagglutinating activity to a considerable degree, while gliadin, in the amounts tested, has no such property. Since edestin is non-toxic, it would seem, as has been previously observed, that the agglutinating action and toxic action are independent. Further, since washed corpuscles were employed, the action must have been between the edestin and the corpuscles, and since by using a pure preparation of edestin, and thereby excluding all other constituents of the seed, the results indicate that hemagglutination is a property of the seed protein.

In view of the observations of Raubitschek¹ that immune sera, normal sera and also peptone inhibit agglutination, it seemed desirable to test this possible antagonistic action in the case of edestin. The procedure was as follows:

¹ *Centralbl. f. Bakteriöl.*, I Orig., 1908, 45, p. 660.

Experiment 84.—The edestin solution (1:100) was made as in Experiment 82. One per cent suspension of thoroughly washed human red corpuscles. Edestin immune rabbit serum No. 19 (inactivated). Fresh active normal rabbit serum. Ten per cent Witte peptone in 0.9 per cent sodium chlorid solution. Varying amounts of 1.35 per cent sodium chlorid solution and water to make all mixtures uniform in volume (4 c.c.). Requisite amounts of sodium chlorid solution and water were put in tubes first, then serum or peptone, then corpuscle suspension, the tubes shaken and finally the edestin solution was added drop by drop with shaking. Tubes were incubated 2 hrs. at 37.5°, read, placed on ice for 20 hrs. and final reading taken. Both readings were done microscopically.

TABLE 2.
ANTI-HEMAGGLUTINATING ACTION OF SERA AND PEPTONE.

TUBE	EDESTIN 1:100 (c.c.)	HUMAN BLOOD COR- PUSCLES 1% (c.c.)	EDESTIN IMMUNE SERUM (c.c.)	NORMAL RABBIT SERUM ACTIVE (c.c.)	PEPTONE 10%* (c.c.)	SALT 1.35% (c.c.)	WATER (c.c.)	AGGLUTINATION	
								2 Hrs. 37.5 C.	22 Hrs. 16° C.
1.....	0.5†	1.0	0.1			2.0	0.4	0	0
2.....	"	"	0.05			"	0.45	0	0
3.....	"	"	0.01			"	0.49	+	+
4.....	"	"	0.005			"	0.5	+	+
5.....	"	"	0.001			"	0.5	++	++
6.....	"	"	0.0005			"	0.5	+++	+++
7.....	"	"	0.0001			"	0.5	+++	+++
8.....	"	"		0.5		"		+++	+++
9.....	"	"		0.1		"	0.4	+	+
10.....	"	"		0.05		"	0.45	+	+
11.....	"	"		0.01		"	0.49	+	+
12.....	"	"			0.5	"		0	0
13.....	"	"			0.25	"	0.25	=	+
14.....	"	"			0.1	"	0.4	+	++
15.....	"	"				0.9% NaCl	0.5	++++	++++
16.....		"	0.1			2.9		0	0
17.....		"		0.5		2.5		++	++
18.....		"			0.5	2.5		0	0
19.....		"				2.0	1.0	0	0

* 10 per cent in salt

† 5 milligrams

The results of the above experiment confirm those of Raubitschek.¹ Edestin immune serum in as small a quantity as 0.05 c.c. completely prevents the agglutination of red cells by five milligrams edestin, under the conditions noted. Indications of this antagonistic action are observed with even smaller amounts of immune serum. In the case of normal serum there was little inhibition. Fifty milligrams of Witte's peptone completely inhibited agglutination. It should be mentioned here that both edestin and gliadin in the concentration employed are precipitated in the presence of red cells and also by the larger amounts of serum. That the agglutinating action of edestin is independent of the mechanical sedimen-

¹ *Wien. klin. Wchnschr.*, 1909, 22, p. 1065; *ibid.*, 1909, 22, p. 1752.

tation is shown by the fact that gliadin produces a heavy precipitation but no agglutination, and that five milligrams of edestin in the presence of immune serum, while precipitating, exhibit no agglutinating activity. It would seem, therefore, that edestin possesses an affinity for red blood corpuscles from both sheep and man which results in the agglutination of the cells, but that this affinity is not as strong as is that between edestin and its immune serum or Witte's peptone.

PRECIPITINS.

The desirability of determining the precipitin reactions of the vegetable proteins has naturally presented itself to many investigators. Experiments to this end have been carried out with edestin, and, among the various reported observations, those of Obermayer and Pick¹ are particularly applicable here. These authors were able to demonstrate specific precipitins in the serum of animals which had received edestin injections. A successful technic for measuring the precipitin potency of an immune serum for edestin requires a nice adjustment of the balance between the antigen and serum. Edestin solutions of a higher concentration than 1:1000 precipitate spontaneously on standing, and immediately when brought in contact with diluted (1:10) normal serum. However, if the edestin be used in a 1:10,000 dilution (prepared as described in Experiment 82) there is no spontaneous sedimentation, and the addition of dilute normal serum (1:10) produces no precipitation.

Experiment 71.—The edestin immune serum was obtained as follows: A large rabbit was given intravenous injections of 0.050, 0.100, 0.050 and 0.150 gram edestin at intervals of 5, 6 and 1 days respectively. The animal was exsanguinated 19 days later, the serum centrifuged and inactivated by heating 15 min. at 56° C. A 1:1000 edestin solution was prepared as already described and 1 c.c. of this was added to 9 c.c. of isotonic salt solution. This solution remains clear for several hours.

The results of the experiment show that edestin immune serum gives a marked precipitate with edestin, while twice the amount of serum produces no precipitation with the same amount of gliadin. Controls with normal serum were negative with both edestin and gliadin. The outcome of this experiment would seem to hold out the promise of affording a reliable method for determining

¹ *Wien. klin. Wchnschr.*, 1904, 17, p. 265; *ibid.*, 1906, 19, p. 324.

TABLE 3.
PRECIPITIN REACTIONS.

TUBE	EDESTIN 1:10000 (c.c.)	GLIADIN 1:10000 (c.c.)	EDESTIN IMMUNE SERUM IN- ACTIVATED 1:10 (c.c.)	NORMAL RABBIT SERUM IN- ACTIVATED (c.c.)	DILUENT NaOH N/1000 (c.c.)	PRECIPITATION	
						2 Hrs. 37° C.	24 Hrs. 16° C.
1.....	1.0	...	1.0	...	...	++++	++++
2.....	1.0	...	0.5	...	0.5	++	++++
3.....	1.0	...	...	1.0	...	+	o
4.....	1.0	...	...	0.5	0.5	o	o
5.....	1.0	...	...	...	1.0	o	o
6.....	...	1.0	1.0	...	...	o	o
7.....	...	1.0	0.5	...	0.5	o	o
8.....	...	1.0	...	1.0	...	o	o
9.....	...	1.0	...	0.5	0.5	o	o
10.....	...	1.0	...	...	1.0	o	o
11.....	...	...	1.0	...	1.0	o	o
12.....	...	...	...	1.0	1.0	o	o

the presence of specific precipitins in the sera of animals immunized with vegetable proteins.

COMPLEMENT DEVIATION.

Dunbar,¹ apparently, is the only observer who has employed the complement deviation method in the differentiation of vegetable proteins. He was able to differentiate the various pollen proteins, and also to distinguish the pollen protein from proteins contained in other parts of the same plant. A search of the literature has failed to reveal a record of any similar experiments with purified vegetable proteins.

Experiment 80.—The test was carried out according to the original Wassermann method with such modifications as the conditions demanded. The usual reagents were used in one-half the quantity specified in the original procedure.

1. The antigen. 0.100 gram edestin suspended in water, 0.8 c.c. N/10 sodium hydrate added and the volume made to 100 c.c. with N/1000 sodium hydrate. One part of this solution was diluted with nine parts of 0.9 per cent sodium chlorid solution giving a 1:10,000 solution of edestin. The gliadin was made in precisely the same manner.
2. Edestin immune serum. This serum was the same as already described (Experiment 71, precipitin test). Inactivated 15 min. at 56° C.
3. Normal rabbit serum. Inactivated 15 min. at 56° C.
4. Hemolytic amboceptor. Immune serum from a rabbit immunized in the usual way with washed sheep's corpuscles. Titre, 0.075 c.c. of a 1:200 dilution.
5. Complement. Pooled, fresh serum, centrifuged, from two or more normal guinea-pigs of about 300 gms. weight. One part of serum diluted with 9 parts of isotonic sodium chlorid solution.

¹ *Ztschr. f. Immunitätsf.*, 1910, 4, p. 740.

TABLE 4.

A. COMPLEMENT DEVIATION WITH EDESTIN.

Tube	Edestin 1:10000 (c.c.)	Edestin Immune Serum 1:10 (c.c.)	Comple- ment 1:10 2 Units (c.c.)	Salt 0.9% (c.c.)	SRBC Sheep (c.c.)	1 Hr. 37° C.	On Ice Over Night
1.....	0.5	0.25	0.5	0.25	1.0	++++	++++
2.....	0.4	0.25	0.5	0.35	1.0	++++	++++
3.....	0.3	0.25	0.5	0.45	1.0	++++	++++
4.....	0.2	0.25	0.5	0.55	1.0	++++±	++++±
5.....	0.1	0.25	0.5	0.65	1.0	+++	+++
6.....	0.05	0.25	0.5	0.7	1.0	++	++
7.....	0.025	0.25	0.5	0.725	1.0	+	+
8.....	0.01	0.25	0.5	0.74	1.0	0	0
Normal Rab- bit Serum							
9.....	0.5	0.25	0.5	0.25	1.0	0	0
10.....	0.4	0.25	0.5	0.25	1.0	0	0
11.....	0.3	0.25	0.5	0.45	1.0	0	0
12.....	0.2	0.25	0.5	0.55	1.0	0	0
13.....	0.1	0.25	0.5	0.65	1.0	0	0
14.....	0.05	0.25	0.5	0.7	1.0	0	0
15.....	0.025	0.25	0.5	0.725	1.0	0	0
16.....	0.01	0.25	0.5	0.74	1.0	0	0
Controls							
17.....	1.0		0.5		1.0	0	0
18.....	1.0		1.0		RBC 0.5	++++	++++
19.....		E. Im. S. 0.5	0.5	0.5	SRBC 1.0	0	0
20.....		E. Im. S. 0.5		1.5	RBC 0.5	++++	++++
21.....		Norm. S. 0.5	0.5	0.5	SRBC 1.0	0	0
22.....		Norm. S. 0.5		1.5	RBC 0.5	++++	++++
23.....			0.5	1.0	SRBC 1.0	0	0

B. GLIADIN. COMPLEMENT DEVIATION WITH EDESTIN IMMUNE SERUM No. 19.

Tube	Gliadin 1:10000 (c.c.)	Edestin Immune Serum 1:10 (c.c.)	Comple- ment 1:10 (c.c.)	Salt 0.9% (c.c.)	SRBC Sheep	1 Hr. 37.5	On Ice Over Night
24.....	0.5	0.25	0.5	0.25	1.0	0	0
25.....	0.25	0.25	0.5	0.5	1.0	0	0
26.....	0.1	0.25	0.5	0.65	1.0	0	0
27.....	0.5	0.25	0.5	0.7	1.0	0	0
28.....	0.025	0.25	0.5	0.725	1.0	0	0
29.....	0.01	0.25	0.5	0.74	1.0	0	0
Normal Rab- bit Serum							
30.....	0.5	0.25	0.5	0.25	1.0	0	0
31.....	0.25	0.25	0.5	0.5	1.0	0	0
32.....	0.1	0.25	0.5	0.65	1.0	0	0
33.....	0.05	0.25	0.5	0.7	1.0	0	0
34.....	0.025	0.25	0.5	0.725	1.0	0	0
35.....	0.01	0.25	0.5	0.74	1.0	0	0
Controls							
36.....	0.5		0.5	0.5	1.0	0	0
37.....	0.5			1.5	RBC 0.5	++++	++++

++++ complete inhibition—no hemolysis.

+++ nearly complete inhibition.

++ some inhibition.

+ slight inhibition.

± trace of inhibition.

0 no inhibition—complete hemolysis.

RBC red blood corpuscles.

SRBC sensitized red blood corpuscles.

6. Sheep's corpuscles. Freshly obtained and thoroughly washed sheep's corpuscles, 5 per cent suspension in isotonic salt solution.
7. Sensitized sheep's corpuscles. A mixture of the 5 per cent suspension of sheep cells, hemolytic amboceptor serum and isotonic sodium chlorid solution was made so that 1 c.c. of the mixture contained 0.5 c.c. of the corpuscle suspension and two units of amboceptor. The mixture was incubated one-half hour at 37.5° and then added to the proper tubes as indicated in the table.

The hemolytic amboceptor and complement were always titrated just previous to the actual test.

The antigen-edestin immune serum and antigen-normal serum mixtures, the anti-complementary, and hemolytic controls were incubated for 1 hr. in the water bath at 37.5°, the sensitized corpuscles then added and the tubes again incubated. Readings were taken at the end of 2 hrs. incubation, the tubes placed in the ice-box over night and a final reading taken.

The results obtained by the above method are sharp and clear. Both edestin and gliadin in three times the amounts used were neither anticomplementary nor hemolytic. Minute amounts of edestin in the presence of edestin immune serum completely bound complement, while a larger amount of gliadin showed no such action. The experiment, therefore, shows that an antigen consisting solely of a pure protein can absorb amboceptor in the serum of a rabbit immunized with that particular protein, and that this combination can deviate complement. The negative action of gliadin with edestin immune serum suggests that this mechanism is specific, but it will be necessary to include other vegetable proteins and other vegetable protein anti-sera before the actual specificity of the reaction can be established.

ANAPHYLAXIS.

The vast literature on anaphylaxis contains few references to the application of this method to the study and differentiation of vegetable proteins. Among these few references, which include observations on sensitization with watery and saline vegetable extracts, there are only two investigations which have to do with isolated proteins. Rosenau and Anderson¹ employed preparations of edestin and excelsin, but failed to obtain any significant results. Several factors were responsible for this failure. They used solutions of these proteins made in 10 per cent sodium chlorid, the intoxicating doses were insufficient and were given subcutane-

¹ *Bull. No. 45, Hyg. Lab., U.S. Pub. Health and Mar.-Hosp. Serv., Wash., 1908, p. 13.*

ously. The work of Wells and Osborne represents the first and only systematic study of pure vegetable proteins as to their anaphylactic reactions. Their preparations were as pure as can be obtained by present methods and were used for the injections dissolved in one-tenth per cent sodium hydrate, thus insuring an adequate intoxicating dose of the protein and obviating the antagonistic action of the hypertonic content of sodium chlorid. All injections were made into the peritoneal cavity. The authors state that because of the tendency of vegetable proteins to cause agglutination of erythrocytes, and because their proteins were in alkalin solution, no intravascular injections were made. Unusual care was taken to control all experimental factors. They used the following preparations: globulin from the castor bean, flax seed, and squash seed; edestin from hemp seed, excelsin from the Brazil nut; proteins from the cocoanut, legumin and vicilin from peas, legumin from the vetch, vignin from the cowpea, glycinin from soy-bean, gliadin from wheat flour and rye, hordein from barley flour, and zein from maize. It was found that all these proteins cause typical anaphylaxis reactions in sensitized animals. The minimum doses which produce sensitization and the time of incubation are about the same as with animal proteins, but as a rule the symptoms are of somewhat slower onset and less stormy course than are those obtained with foreign sera, and the minimum intoxicating doses are larger. There are also considerable differences in the toxicity of the several proteins to sensitized animals. The most toxic proteins, as measured by the frequency of severe and fatal reactions, usually caused death when given in 0.1 gm. doses to properly sensitized animals. Edestin, it is interesting to note in the present connection, caused the least severe reactions of any of the proteins. Marked specificity of reaction was shown, within certain limits, by the vegetable proteins employed.

In the present study the same two preparations of edestin and gliadin used by Wells and Osborne were employed. They were dissolved in water with just enough N/10 sodium hydrate to effect solution and therefore were somewhat less alkalin than the solutions of these authors. Sensitizing injections were always intraperitoneal

but the intoxicating doses were always given intravenously. It was found that at least 40 or 60 milligrams of these proteins in faintly alkaline solution could be injected into the jugular vein of 200-gm. guinea-pigs without causing any appreciable symptoms. Whatever hemagglutinating action these solutions exerted in no way interfered with or complicated the hypersensitive reaction. Guinea-pigs of about 200 gms. in weight were always used unless otherwise stated. All glass syringes were used, one set with needles for edestin and another for gliadin. These were thoroughly cleansed and dried before use.

Sensitizing and intoxicating doses.—Wells and Osborne found that five ten-thousandth milligram (0.000005 gm.) of squash globulin effectively sensitized a guinea-pig, while one ten-thousandth milligram (0.000001 gm.) of edestin rendered a pig sufficiently sensitive to react with moderate symptoms to an intoxicating dose of 0.1 gm. edestin. The intoxicating power, however, is much less than that of egg albumin, serum proteins, etc., for the minimum intoxicating dose of their preparation of edestin was two to five milligrams, while severe reactions were not obtained with less than 20 to 40 milligrams. The intraperitoneal route of administering the intoxicating dose undoubtedly is accountable for the comparatively large amounts required to produce severe or fatal reactions. Edestin and gliadin are precipitated immediately when brought in contact with serum or tissues, hence their absorption from the peritoneal cavity must be too slow to produce the typical fulminating intoxication unless given in comparatively large amounts.

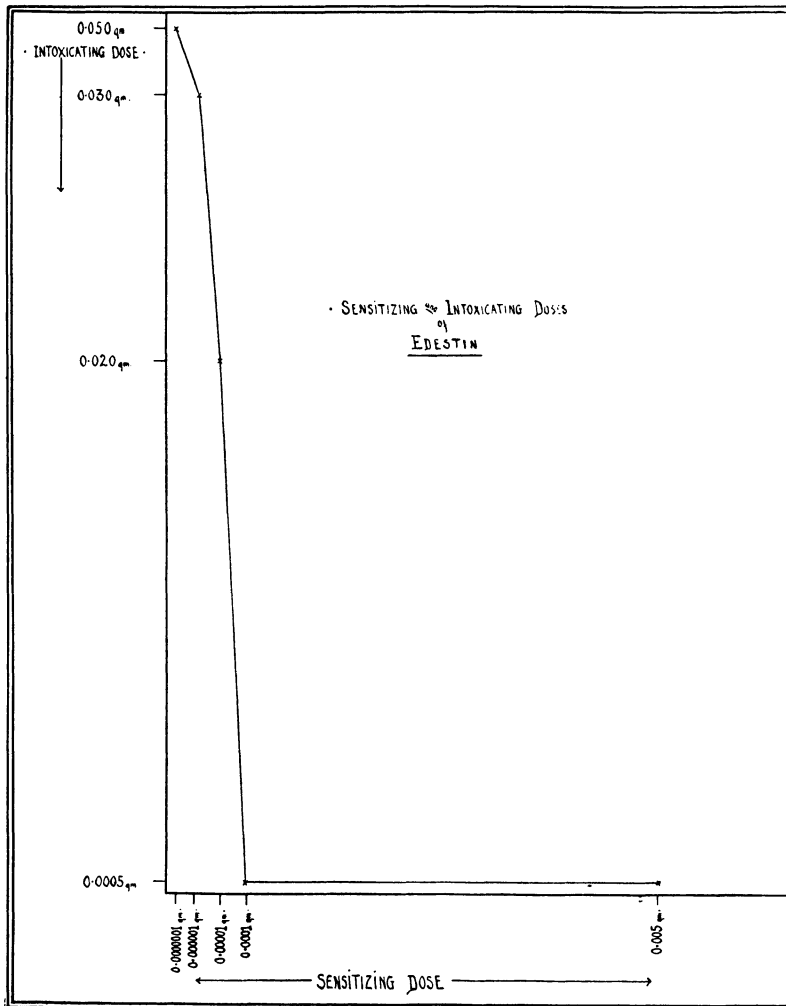
Experiment 73.—The solutions used for the sensitizing injections were made as follows: One gram edestin was suspended in a small amount of water, enough N/10 sodium hydrate added to effect solution and the volume then made up to 1 litre with N/1000 sodium hydrate. Further solutions to contain 0.1, 0.01, 0.001 and 0.0001 milligrams in 1 c.c. were made from this original solution by diluting the required amount of the 1:1000 solution to 1 litre with N/1000 sodium hydrate. The dilutions were always made in this large volume to reduce the factor of error in weighing and diluting. All dilutions were made in volumetric flasks with the N/1000 sodium hydrate at the required temperature. The indicated amounts of edestin were injected in 1 c.c. volume. The intoxicating solutions were usually made up to contain 0.020 gram edestin or gliadin in 1 c.c. Sensitizing dose, intraperitoneally, intoxicating dose, intravenously.

TABLE 5.
 TITRATION OF SENSITIZING AND INTOXICATING DOSES OF EDESTIN.

PIG	SENSITIZING DOSE		INTERVAL (Days)	INTOXICATING DOSE		SYMPTOMS	AUTOPSY	
	Amount (Milligrams)	Volume (c.c.)		Amount (Milligrams)	Volume (c.c.)			
Series A {	723.....	0.001	1.0	21	20	1.0	+ R	
	732.....	0.001	1.0	21	30	1.5	+ R	
	733.....	0.001	1.0	21	40	2.0	+ R	
	794.....	5.0	1.0	21	30	1.5	+++R	
Series B {	810.....	0.0001	1.0	21	40	2.0	++R	
	811.....	0.0001	1.0	21	50	2.5	+++D 4'	Typical
	807.....	0.0005	1.0	21	50	2.5	+++D 2'30"	Typical
	734.....	0.001	1.0	21	20	1.0	++ R	
	730.....	0.001	1.0	21	30	1.5	+++D 3'30"	Typical
	728.....	0.001	1.0	21	40	2.0	+++D 4'30"	Typical
	731.....	0.001	1.0	21	60	3.0	+++D 3'	Typical
	738.....	0.01	1.0	21	20	1.0	+++D 3'30"	Typical
Series C {	756.....	0.1	1.0	21	0.1	1.0	++ R	
	755.....	0.1	1.0	21	0.25	2.5	++ R	
	750.....	0.1	1.0	21	0.5	5.0	+++D 2'	Typical
	752.....	0.1	1.0	21	0.5	0.5	+++D 5'30"	Typical
Series D {	757.....	0.1	1.0	21	1.0	1.0	+++D 6'	Typical
	753.....	0.1	1.0	21	5.0	0.5	+++D 4'	Typical
	754.....	0.1	1.0	21	10	1.0	+++D 4'30"	Typical
Series E {	788.....	5.0	1.0	21	0.1	1.0	++ R	
	784.....	5.0	1.0	21	0.5	1.0	+++D 3'30"	Typical
	789.....	5.0	1.0	21	30	1.5	+++D 4'	Typical

This experiment seems to establish the minimum sensitizing dose of edestin rendering subsequent fatal intoxication possible as one ten-thousandth milligram, and the minimum fatal intoxicating dose as one-half milligram (0.0005 gm.). Apparently ascending amounts of edestin, within certain limits, induce ascending degrees of sensitization, since 50 milligrams (0.050 gm.) edestin were required to intoxicate the animals sensitized with one ten-thousandth milligram (0.000001 gm.) while one-half milligram (0.0005 gm.) was the minimum dose for pigs sensitized with one-tenth to five milligrams (0.0001 to 0.005 gm.) edestin. In the present investigation it has been found that guinea-pigs react uniformly and that, when suitable sensitizing and intoxicating doses of edestin are used and the intoxicating injections given intravenously, fatal reactions take place in 100 per cent of the sensitive animals. The minimum fatal intoxicating dose, when given intravenously, is one-fortieth to one-eightieth of the fatal intraperitoneal dose reported by Wells and Osborne.

TABLE 6.



In Series A an interesting point developed. By mistake some edestin was used for the intoxicating solutions which had been dried for four hours at 103° . Although the pigs in this series developed more or less marked symptoms, none died, while pigs similarly sensitized in Series B and E reacted fatally when unheated edestin was used.

Specificity.—Wells and Osborne state that animals sensitized with edestin possessed no sensitiveness to their preparations of

castor bean globulin and squash seed globulin. In the present study animals sensitized with edestin were tested with squash seed globulin, castor bean globulin, flaxseed globulin, and corylin, a globulin from the hazelnut. The three first named globulins were suggested by Dr. Osborne as being chemically and physically more nearly like edestin than any other known vegetable proteins.

Experiment 87.—The globulins were dissolved in the manner already described for edestin, and all gave clear solutions. The squash seed globulin required about four times the amount of alkali used to dissolve the other globulins, as this preparation contained a larger proportion of the insoluble form of the globulin than did the other preparations.

TABLE 7.
SPECIFICITY OF EDESTIN SENSITIZATION.

Pig	Sensitizing Dose Edestin (Milligrams)	Interval (Days)	Intoxicating Dose Globulin (Milligrams)	Symptoms	Autopsy	Interval (Hrs.)	Second Intoxicating Dose Edestin (Milligrams)	Symptoms	Autopsy
760...	5.0	41	40 gliadin	OR		..	..		
759...	5.0	41	60 "	OR		..	..		
775...	5.0	45	40 hazelnut	OR		..	..		
762...	5.0	45	60 "	OR		48	1.0	+++D 6'30"	Typical
769...	5.0	45	40 squash seed	OD in night		..	..		
773...	5.0	45	60 "	OD in night		..	..		
783...	5.0	45	40 cotton seed	OR		48	1.0	+++D 3'	Typical
761...	5.0	45	60 "	OR		..	..		
778...	5.0	45	20 flaxseed	++R		48	1.0	+R	
777...	5.0	45	60 "	+++D 3'30"	Typical	..	..		
820...	...	..	60 "	OR		..	..		

Pigs 769 and 773 showed no anaphylactic symptoms but died within 12 hours. Death was probably caused by the greater alkalinity of the squash seed globulin solution. However, the small number of animals used permits of no definite conclusions. Apparently edestin fails to sensitize animals to globulins from the squash seed, castor bean and the hazelnut. Two animals sensitized with edestin reacted with typical anaphylactic symptoms, one fatally, to flaxseed globulin.¹

Inheritance of sensitiveness.—It is known that guinea-pigs born of a mother previously sensitized to a given protein inherit a certain degree of sensitiveness to that protein. The following observation is recorded here in order to illustrate this point and to show that this rule holds good in the case of edestin.

¹ This cross-reaction is merely recorded here since the specificity of the anaphylactic reactions in the case of pure vegetable proteins is being systematically investigated by Wells and Osborne.

Experiment 62.—An adult female pig received intraperitoneal injections of 0.005, 0.010, and 0.015 gm. edestin on Aug. 20, 24, and 31 respectively, being pregnant at the time. The mother's sensitiveness was shown by the fact that seven other pigs sensitized with the same amounts at the same time all reacted fatally to 0.005 gm. or more of edestin 21 days after the last sensitizing injection. On Sept. 27, 27 days after the last injection she gave birth to two young, Nos. 534-1 and 534-2. On Nov. 19, when 53 days old, they were tested as follows:

TABLE 8.

	Edestin i. v.	Symptoms	Autopsy
534-1.....	0.050 gm.	Immediate, typical Death, 2 minutes	Typical
534-2.....	0.030 gm.	Immediate, typical Recovery	

Table 8 shows that both animals had inherited the mother's sensitiveness to edestin, but that they possessed it to a considerably less degree.

Passive sensitization.—Since it had been shown in the present investigation that the serum of a rabbit receiving repeated intravenous injections of edestin contains precipitating and complement deviating antibodies for edestin (Experiments 71 and 80) the endeavor was made to demonstrate the presence of an anaphylactic antibody in the same serum. It is now a well-established fact that the serum of an animal (especially the rabbit), sensitized with any protein, has the property of passively sensitizing a guinea-pig to that protein. When the proper conditions, such as the amount of serum injected, the time interval and size of the intoxicating dose, are fulfilled, the transference of sensitiveness is usually successful.

Experiments 63 and 72.—Three different edestin immune rabbit sera were used, Nos. 7, 19 and 111, the method of obtaining which has already been described. Serum No. 7 was used 24 hrs. after bleeding and was not inactivated. Sera No. 19 and 111 were inactivated by heating for 15 min. at 56°. The serum, with one exception (Pig 640), was injected intraperitoneally, and the intoxicating injection of edestin made the following day, intravenously. (See Table 9, p. 118.)

The minimum sensitizing dose, therefore, of sera No. 19 and No. 111 was between 0.05 and 0.10 c.c., although some pigs reacted fatally when passively sensitized with 0.01 and 0.025 c.c. of serum No. 111.

TABLE 9.

PASSIVE SENSITIZATION: TITRATION OF SENSITIZING DOSE OF IMMUNE SERUM.

PIG	IMMUNE SERUM		INTERVAL (Hrs.)	INTOXICATING DOSE		SYMPTOMS	AUTOPSY
	Quantity (c.c.)	No.		Edestin (gms.)	Vol. (c.c.)		
640.....	5.0	7	27	0.050	2.5	+++D 3'	Typical
641.....	5.0	7	27	0.040	2.0	+++D 3'	"
700.....	4.0	111	27½	0.040	2.0	+++D 3'15"	"
701.....	2.0	111	27½	0.040	2.0	+++D 3'45"	"
702.....	1.0	111	18	0.040	2.0	+++D 3'30"	"
703.....	0.5	111	18	0.040	2.0	+++D 2'30"	"
704.....	0.25	111	18	0.040	2.0	+++D 3'	"
717.....	0.05	111	19	0.030	1.5	+++R	
718.....	0.05	111	19	0.020	2.0	+++D 4'30"	Typical
719.....	0.025	111	19	0.015	1.5	+ R	
720.....	0.025	111	19	0.025	1.25	+++D 4'	Typical
721.....	0.01	111	19½	0.025	1.25	+++D 3'30"	Partly inflated
724.....	0.01	111	24	0.020	1.0	++ R	
718x.....	0.1	19	24	0.040	2.0	+++D 3'30"	Typical
719x.....	0.05	19	24	0.040	2.0	+++D 3'30"	"
720x.....	0.05	19	24	0.040	2.0	++ R	
721x.....	0.01	19	24	0.040	2.0	o R	
722x.....	0.01	19	24	0.020	1.0	o R	

Experiment 79.—In order to compare the degree of sensitiveness produced by active and by passive sensitization the following experiment was carried out: (For titre of intoxicating dose in actively sensitized animals see Tables 5 and 6, Experiment 73). 0.1 c.c. of Serum No. 19, representing twice the minimum sensitizing dose, was injected intraperitoneally, and the intoxicating dose of edestin given intravenously on the following day.

TABLE 10.

TITRATION OF INTOXICATING DOSE OF EDESTIN IN PASSIVELY SENSITIZED ANIMALS.

PIG	EDESTIN IMMUNE SERUM No. 19 (c.c.)	INTERVAL (Hrs.)	INTOXICATING DOSE		SYMPTOMS	AUTOPSY
			Amount (gms.)	Vol. (c.c.)		
704.....	0.2	24	0.000025	0.25	o R	
703.....	0.2	24	0.00005	5.0	o R	
702.....	0.2	24	0.0001	1.0	+++D 3'30"	Typical
786.....	0.2	24	0.00025	0.25	+++D 4'30"	"
785.....	0.2	24	0.001	1.0	+++D 3'30"	"
787.....	0.2	24	0.0025	2.5	+++D 3'30"	"
790.....	0.2	24	0.010	1.0	+++D 3'30"	"
791.....	0.2	24	0.040	4.0	+++D 3'30"	"
789.....	0.2	24	0.015 i.p.	1.5	o R	

Guinea-pigs passively sensitized with 0.1 c.c. of edestin immune serum reacted fatally to one-tenth milligram (0.0001 gram) edestin or one-fifth of the minimum amount required to intoxicate an actively sensitized pig.

The poisonous substance obtained in the hydrolysis of edestin by the method of Vaughan.—In a previous communication the authors¹ described the preparation and action of a poisonous substance obtained from the tubercle bacillus by boiling dried alcohol and ether extracted tubercle bacilli with two per cent sodium hydrate in absolute alcohol. A similar hydrolysis by the Vaughan method was performed with the preparation of edestin supplied by Dr. Osborne. The residue obtained from the alkaline alcoholic fraction, after neutralization, filtration and evaporation, was purified by redissolving four times in absolute alcohol, filtering and evaporating *in vacuo*. The final yellowish powder was exceedingly hygroscopic, soluble in water and in alcohol. Its properties and action were similar so far as tested with those already described for the poisonous product from the tubercle bacillus.

Experiment 48.—The preparation was dried over phosphorous pentoxide and sulfuric acid in a vacuum desiccator, (pressure less than 1 mm. of mercury). The poison was dissolved in isotonic salt solution and injected intravenously.

TABLE 11.
TITRATION OF POISONOUS SUBSTANCE FROM EDESTIN.

GUINEA-PIG		INJECTION OF POISON			SYMPTOMS	AUTOPSY
No.	Weight	Amount (gms.)	(c.c.)	Relation to Body Weight		
695.....	215	0.0215	2.15	1:10000	+++D 3'30"	Typical
696.....	230	0.020	2.0	1:11500	+++D 3'30"	"
697.....	240	0.012	1.2	1:20000	+++D 4'45"	"
698.....	245	0.0082	0.82	1:30000	+++D 3'30"	"
699.....	245	0.0061	0.61	1:40000	+++D 4'15"	"
700.....	230	0.0046	0.46	1:50000	++ R	

One part of this poisonous hydrolytic product from edestin to 40,000 parts of guinea-pig produced an acute fatal intoxication indistinguishable from anaphylactic shock. The possible relationship of this poison to the hypothetical anaphylatoxin formed in the sensitive animal and the so-called anaphylatoxin of Friedberger produced in the test tube, is discussed in the above cited communication. The chemical nature of this substance is being investigated.

Anaphylatoxin.—Friedberger observed that if fresh guinea-pig complement is allowed to act upon bacteria which have previously

¹ *Jour. Med. Research*, 1912, 26, p. 317.

been in contact with their specific immune serum, a substance is formed which produces the characteristic symptoms of anaphylactic shock. He considered that this phenomenon was due to a digestive action by the complement on the antigen-amboceptor combination and that this action *in vitro* was identical with the anaphylactic reaction *in vivo*. The hypothesis has been subjected to much criticism based upon a variety of experimental data. It has been objected that bacterial cultures and sera contain other substances, which when subjected to the action of complement might yield toxic substances. It has also been shown that complement alone, without the intervention of immune serum, is able to transform bacterial and serum protein into poisonous products, while Besredka¹ claims that the poison arises in the action of complement on the peptone of the medium on which the bacteria are grown. More recently Rosenow² has shown that the autolyzate of virulent pneumococci, when injected into guinea-pigs, calls forth a fulminating anaphylactoid reaction. A similar reaction is also produced by extracts of typhoid bacilli. In view of this confusing diversity of opinion it seemed desirable to attempt the production of the so-called anaphylatoxin from edestin by the method of Friedberger. The use of edestin as antigen would exclude the other components of bacterial masses, of sera and other protein mixtures previously used in this connection. Edestin is non-toxic in itself. Further, it is a substance which bears no near biological relationship to the constituents of guinea-pig tissues, and as edestin can be absolutely excluded from the food of guinea-pigs, it is most unlikely that they should possess any natural amboceptors for this protein. The possession of a potent immune serum, not only containing precipitating and complement deviating antibodies but also having the property of passively sensitizing guinea-pigs in small doses, supplied another desirable factor.

Preliminary experiments were carried out, using comparatively concentrated solutions of edestin and undiluted serum. A heavy precipitate immediately resulted and it was felt that this rapid precipitation might hinder the proper absorption of amboceptor by the edestin. Hence, in the later experiments (Experiments 85 and 86), the edestin and serum were employed in dilutions which

¹ *Ztschr. f. Immunitätsf.*, 1913, 16, p. 249.

² *Jour. Infect. Dis.*, 1912, 11, p. 235.

SOME IMMUNITY REACTIONS OF EDESTIN

121

yielded precipitates only after incubation and standing at room temperature.

TABLE 12.
ANAPHYLATOXIN. A. TITRATION OF ANTIGEN.
(EXPERIMENT 64.)

PIG	EDESTIN		EDESTIN IMMUNE SERUM No. 7 (c.c.)	CONTACT 37.5° 16.0° (Hrs.)		COMPLE- MENT (c.c.)	INCUBATION TIME 37.5° (Hrs.)	SYMPTOMS
	Amount (gms.)	Vol. (c.c.)						
642.....	0.050	2.0	2.0	4	20	3.0	6	o
643.....	0.025	2.0	2.0	4	20	3.0	6	o
644.....	0.010	2.0	2.0	4	20	3.0	6	o
645.....	0.005	2.0	2.0	4	20	3.0	6	++R
650.....	0.005	2.0	2.0	22	..	3.0	6	++R
651.....	0.0025	2.0	2.0	22	..	3.0	6	++R
652.....	0.001	2.0	2.0	22	..	3.0	6	o
646.....	0.050	2.0	..	..	..	3.0	6	o
647.....	0.025	2.0	..	..	..	3.0	6	o
648.....	0.010	2.0	..	..	..	3.0	6	o
600.....	0.005	2.0	..	..	..	3.0	6	o
653.....	0.005	2.0	..	..	..	3.0	6	o
654.....	0.0025	2.0	..	..	..	3.0	6	o
655.....	0.001	2.0	..	..	..	3.0	6	o

TABLE 13.
ANAPHYLATOXIN. B. DETERMINATION OF INCUBATION TIME.
(EXPERIMENT 70.)

PIG	EDESTIN		EDESTIN IMMUNE SERUM No. 111 (c.c.)	CONTACT 37.5° 16.0° (Hrs.)		COMPLE- MENT (c.c.)	INCUBA- TION TIME 37.5°C (Hrs.)	SYMPTOMS	AUTOPSY
	Amount (gms.)	Vol. (c.c.)							
700.....	0.005	2.0	2.0	3	21	4.0	4	o	
710.....	0.005	2.0	2.0	3	21	4.0	6	++R	
711.....	0.005	2.0	2.0	3	21	4.0	8	OR	
712.....	0.005	2.0	2.0	3	21	4.0	12	+D 8 hrs	Typical
713.....	0.005	2.0	..	..	..	4.0	4	o	
714.....	0.005	2.0	..	..	..	4.0	6	o	
715.....	0.005	2.0	..	..	..	4.0	8	o	
716.....	0.005	2.0	..	..	..	4.0	12	o	

TABLE 14.
ANAPHYLATOXIN. C. DETERMINATION OF PROPER AMOUNT OF IMMUNE SERUM.
(EXPERIMENTS 85 AND 86.)

PIG	EDESTIN		EDESTIN IMMUNE SERUM No. 19		CONTACT 37.5° 16.0° (Hrs.)		COMPLE- MENT (c.c.)	INCUBA- TION TIME 37.5°C (Hrs.)	SYMPTOMS	AUTOPSY
	Amount (gms.)	Vol. (c.c.)	Amt. (c.c.)	Vol. (c.c.)						
800..	0.001	10	1.0	10	2	22	4.0	6	o	
801..	0.002	10	0.5	5	2	22	4.0	6	++R	
802..	0.002	10	1.0	10	2	22	4.0	6	+R	
803..	0.002	10	2.5	25	2	22	4.0	6	+++R	
816..	0.005	5	3.0	30	2	22	5.0	6	++R	
804..	0.002	10	5.0	50	2	22	5.0	6	+++D 4'30"	Typical
819..	0.002	10	5.0	50	2	40	5.0	6	+++D 4'	"
817..	0.002	0.2	..	..	..	..	5.0	6	o	
818..	0.005	0.5	..	..	..	..	5.0	6	o	

Cultural tests made on the contents of the various tubes showed them to be sterile.

The above experiments show that when fresh complement is allowed to act for a definite time at 37.5° upon the precipitate obtained by mixing proper amounts of edestin and edestin immune serum, a soluble substance or substances are formed which produce the typical fatal intoxication and gross pathological appearance of true anaphylaxis, and that this substance is not formed when complement alone, under identical conditions, is allowed to act upon edestin.

CONCLUSIONS.

1. Crystallized preparations of edestin from hemp seed and gliadin from wheat flour were used, and were specially prepared for anaphylactic experiments by Dr. Thomas B. Osborne. They were dissolved in water with the addition of the smallest possible amount of sodium hydrate necessary to effect solution.

2. *Hemagglutination*.—Edestin, even in small amounts, agglutinates washed red blood corpuscles of the sheep and of man. Both edestin immune serum and peptone completely inhibit this action under the conditions noted. Gliadin, in the amounts used, exerts no agglutinating action.

3. *Precipitins*.—The serum of a rabbit which has been immunized with edestin contains a precipitating antibody for edestin, but none for gliadin in at least twice the concentration giving a positive reaction with edestin.

4. *Complement deviation*.—Edestin, in the presence of edestin immune serum, when both are used in non-anti-complementary and non-hemolytic amounts, completely binds complement. Gliadin, in the presence of edestin immune serum, fails to bind complement.

5. I. *Anaphylaxis*.—(a) Sensitizing dose, 0.0000001 gram edestin, injected intraperitoneally may be considered as the minimum sensitizing dose. Guinea-pigs sensitized with this amount react fatally when a sufficient amount of edestin (0.050 gm.) is injected intravenously after the proper interval. (b) When the sensitizing dose is one-tenth to five milligrams the intravenous injection of one-half milligram produces typical anaphylactic death in from two to six minutes.

II. *Specificity*.—Pigs sensitized to edestin fail to react to intravenous injections of gliadin, or the globulins from squash seed, the castor bean or the hazelnut. Two animals reacted positively, one fatally, when given an intravenous injection of flaxseed globulin. The fatal dose of flaxseed globulin was, however, 40 to 120 times the minimum fatal intoxicating dose of edestin.

III. *Inheritance*.—Guinea-pigs born of a mother sensitized with edestin while pregnant, inherit this sensitiveness but possess it to a somewhat less degree than does the mother.

IV. *Passive sensitization*.—The intraperitoneal injection of edestin immune rabbit serum passively sensitizes the guinea-pig. 0.05 to 0.1 c.c. of two such sera tested, rendered guinea-pigs sufficiently sensitive to react fatally to an intravenous injection of edestin on the following day. The degree of sensitiveness passively conferred appears to be somewhat greater than that induced by active sensitization.

V. *Poisonous hydrolytic product*.—When edestin is hydrolyzed by an alcoholic solution of sodium hydrate according to the method of Vaughan, a substance is formed which produces a fatal intoxication in the guinea-pig apparently identical with true anaphylactic shock. The intravenous injection of one part of this poison to 40,000 parts of guinea-pig by weight constitutes the minimum fatal dose.

VI. *Anaphylatoxin*.—When suitable amounts of edestin and edestin immune serum are allowed to remain in contact for a given length of time, a precipitate is formed, which, when washed with salt solution and mixed with fresh guinea-pig complement and incubated at body temperature, yields a substance or substances, which, when injected into a guinea-pig intravenously, produces a fatal intoxication, apparently identical in every way with the anaphylactic reaction. Fresh complement, when allowed to act under similar conditions with edestin alone, yields no poisonous substance. From edestin, therefore, by the action of immune serum and complement, under the experimental conditions noted, a toxic product is obtained which seems to correspond to the anaphylatoxin of Friedberger.